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Rapid Detection of Bacterial Antibiotic Resistance: Preliminary Evaluation of PCR Assays Targeting Tetracycline Resistance Genes

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ABSTRACT

The rapid spreading of bacterial antibiotic resistance and the lack of methods that would allow fast and accurate determination of the resistance profile of an infectious agent have become a major concern for public health. In the context of biological warfare or a bioterrorist attack, resistance against commonly used antibiotics would severely compromise the capabilities of the responsible agencies to protect large numbers of exposed individuals against a potentially lethal infection. In this study the possibility of developing polymerase chain reaction (PCR) assays for rapid detection of tetracycline resistance genes was evaluated. PCR assays were designed for seven resistance genes that frequently occur in Gram-positive and Gram-negative species and tested on eight tetracycline resistant clinical isolates. Positive results were obtained from seven of the eight species tested.

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Executive Summary

The link between the application of antibiotics and the development of resistance has been well documented since the discovery of penicillin in the 1940s. The excessive use of antibiotics in clinical therapy and in agriculture has caused a rapid spread of resistance in both clinical and environmental isolates. Consequently, determination of the antimicrobial susceptibility of pathogenic bacteria is of vital importance to provide the optimal chemotherapy for infected patients.

Traditional methods for susceptibility testing require isolation of the pathogen from a specimen sample and culturing on appropriate media containing antibiotic(s). These methods are time consuming and labour intensive and require at least 24 to 48 hours to provide a definitive result. Considerably more time is needed for fastidious pathogens such as *Coxiella burnetii*, an organism considered a potential biological warfare/bioterrorism agent. *C. burnetii* cannot be grown in axenic medium. Susceptibility testing has to be done in animals, chicken embryos or cell culture and requires up to 9 days to determine susceptibility.

The scenario of a bioterrorist attack that exposes a large number of individuals to an infectious agent necessitates an immediate response to provide adequate protection against a potentially lethal infection, and rapid determination of antibiotic resistance(s) of the infectious agent is crucial. Application of antibiotics that are ineffective is likely to have disastrous consequences. Tetracycline is a commonly used antibiotic in the management of diseases caused by a number of potential biological warfare agents such as anthrax, cholera, plague, and Q-fever. Infections like inhalation anthrax and pneumonic plague, caused by *Bacillus anthracis* and *Yersinia pestis*, have to be treated with an effective antibiotic within 24 to 48 hours post exposure. In the majority of cases antibiotic treatment is ineffective once symptoms appear.

The polymerase chain reaction (PCR) is a rapid and sensitive tool for the detection of resistance genes. Seven PCR assays for the detection of frequently occurring tetracycline resistance genes in Gram-positive and Gram-negative species were designed and evaluated with eight tetracycline resistant clinical isolates. Putative resistance genes were detected in seven isolates.

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Matthias Dorsch graduated with a diploma from Christian Albrechts University, Kiel, Germany, in 1987 and was awarded a Ph. D. in 1990 from the same University. Topic of the thesis was the phylogeny of Gram-positive eubacteria. He conducted postdoctoral research at The University of Queensland, The University of New South Wales and Macquarie University. During this time he specialised in detection and identification of bacterial and protozoan pathogens and indicator organisms. He joined the Human Protection & Performance Division in 2001. Focus of his research is the development of rapid detection and identification methods for biological warfare agents.

Contents

1. INTRODUCTION	1
2. MATERIALS AND METHODS	5
2.1 Materials	5
2.1.1 Amplification primers.....	5
2.1.2 Bacterial strains and media	5
2.1.3 DNA isolation.....	5
2.1.4 Real-time PCR	5
2.1.5 Conventional PCR	5
2.1.6 Thermal cyclers	5
2.2 Methods	6
2.2.1 Susceptibility testing of strains	6
2.2.2 DNA isolation.....	6
2.2.3 Design of PCR amplification primers	6
2.2.4 Real-time PCR	7
2.2.5 Conventional PCR	8
3. RESULTS AND DISCUSSION.....	9
4. CONCLUSIONS	12
5. BIBLIOGRAPHY	13
6. FIGURES	16
APPENDIX	20

1. Introduction

The discovery and successful application of penicillin, cephalosporins and streptomycin in the 1940s was closely followed by the emergence of antibiotic resistant bacteria. The rapid increase in the number of antibiotic resistant species, including strains that carry multidrug-resistance genes (Fluit *et al.* 1999, 2000), and the lack of methods that would allow rapid and accurate determination of the resistance profile of a pathogenic bacterium have become a severe problem for public health.

Determination of antimicrobial susceptibility of a clinical isolate is in many cases crucial for providing the optimal antimicrobial therapy of infected patients (Fluit *et al.* 2001). The conventional methods for susceptibility testing require the isolation of the pathogen from a clinical specimen and culturing on the appropriate media that contain antibiotic(s). This is typically carried out using micro-diffusion discs that allow determination of susceptibility and the minimum inhibitory concentration (MIC) of an antibiotic that a pathogen is susceptible to. In most cases a definitive result can be obtained within 24 hours but will take longer when fastidious or slow growing pathogens such as certain *Mycobacterium* spp. need to be tested. *Coxiella burnetii*, recognised as a potential biological warfare agent, is an extreme example. The organism cannot be grown in axenic medium. For the purpose of antimicrobial susceptibility testing, three models of infection have been developed, including animals (Huebner *et al.* 1948), chicken embryos (Maurin & Raoult 1993), and cell cultures (Torres & Raoult 1993). The minimum time requirement of these models is between 6 and 9 days.

The fact that antibiotic resistant organisms can easily be generated in the laboratory, either through selective media or by relatively simple genetic engineering techniques, has become a major concern (Courvalin 1995; Hilleman 2002; Sefton 2002). Strains of *B. anthracis* that are resistant to ciprofloxacin, doxycycline and rifampin have been generated by passaging the strains through media containing low levels of the antibiotics (Choe *et al.* 2000; Vogler *et al.* 2002), and Russian scientists have created a *B. anthracis* strain resistant to clindamycin, chloramphenicol, erythromycin, penicillin, rifampin and tetracycline (Stepanov *et al.* 1996).

The potential exposure of a large number of individuals to an infectious agent due to an act of bioterrorism is a drastic scenario which highlights the need for methods that enable rapid determination of bacterial resistance profiles. Authorities involved in such an incident have to take immediate action to protect the victims against a potentially lethal infectious disease even if the infectious agent would not exhibit resistance against commonly used antibiotics. Inhalation anthrax is considered fatal if antibiotic treatment does not commence within 24 – 48 hours post exposure (Henderson, D.A. 1999; Franz *et al.* 1997). Antibiotic treatment is usually ineffective once symptoms appear. Similarly critical is the timing for treatment of pneumonic plague. Antibiotics have to be administered within 24 hours of occurrence of the first symptoms (Franz *et al.* 1997; Gilligan 2002). Pathogens possessing one or even multiple resistances against antibiotics that are normally effective would severely exacerbate the situation as administering ineffective antibiotics may result in mass casualties.

Nucleic-acid targeted detection systems as the polymerase chain reaction (PCR) offer rapid and sensitive methods to detect the presence of resistance genes and are crucial in the

elucidation of resistance mechanisms. They are widely applied in clinical microbiology and are not limited to detection and identification of a target organism but also enable the characterisation of properties of the target, such as virulence factors and antimicrobial resistance (Fluit *et al.* 2001). They are particular useful for investigations in slow-growing or non-culturable organisms.

Tetracycline resistance was chosen as subject of this initial study because tetracycline and its derivatives are the antibiotics of choice for chemotherapy against a number of organisms that are considered potential biological warfare agents (see table 1). Further, the genes coding for different resistance mechanisms exhibit a relatively high degree of homology between different bacterial species, and this may facilitate the design of diagnostic PCR assays.

Table 1. Antibiotics commonly used against diseases caused by potential BW agents

Organism	Disease	Antibiotics
<i>Bacillus anthracis</i>	Anthrax	Ciprofloxacin, Doxycycline
<i>Brucella</i> spp.	Brucellosis	Aminoglycosides, Doxycycline, Tetracyclines
<i>Burkholderia mallei</i>	Glanders	Penicillins, Tetracyclines
<i>Burkholderia pseudomallei</i>	Melioidosis	Penicillins, Tetracyclines
<i>Chlamydia psittaci</i>	Psittacosis	Macrolides, Tetracyclines
<i>Coxiella burnetii</i>	Q-Fever	Doxycycline, Quinolones
<i>Francisella tularensis</i>	Tularemia	Fluoroquinolones, Tetracyclines
<i>Rickettsia prowazekii</i>	Typhus Fever	Chloramphenicol, Tetracyclines
<i>Salmonella</i> spp.	Salmonellosis	Ampicillin, Ciprofloxacin, Gentamycin
<i>Salmonella typhi</i>	Typhoid Fever	Ampicillin, Ciprofloxacin, Gentamycin
<i>Shigella</i> spp.	Shigellosis	Ampicillin, Ciprofloxacin
<i>Vibrio cholerae</i>	Cholera	Tetracyclines, Trimethoprim/Sulfamethoxazole
<i>Yersinia pestis</i>	Plague	Gentamycin, Streptomycin, Tetracyclines

From: <http://www.cdc.gov/>

The first tetracyclines to be discovered in the late 1940s were chlortetracycline and oxytetracycline, metabolites produced by *Streptomyces aureofaciens* and *Streptomyces ramosus*, respectively. Further tetracyclines discovered later were either naturally occurring molecules or semi-synthetic products (Chopra & Roberts 2001). The principal members of the tetracycline class are summarised in table 2. Tetracyclines inhibit bacterial protein synthesis by blocking the bacterial ribosome against binding of the aminoacyl-tRNA (Chopra *et al.* 1992; Schnappinger & Hillen 1996). Several mechanisms that confer resistance to tetracycline and/or its derivatives have been described to date (Chopra & Roberts 2001). An overview of *tet* and *otr* (oxytetracycline) resistance genes described to date is given in table 3.

Table 2. Principal members of the tetracycline class

Generic Name	Trade Name	Year of discovery	Status	Therapeutic administration
Chlortetracycline	Aureomycin	1948	Marketed	Oral
Oxytetracycline	Terramycin	1948	Marketed	Oral and parenteral
Tetracycline	Acromycin	1953	Marketed	Oral
Demethylchlortetracycline	Declomycin	1957	Marketed	Oral
Rolitetracycline	Reverin	1958	Marketed	Oral
Limecycline	Tetralysal	1961	Marketed	Oral and parenteral
Clomocycline	Megaclor	1963	Marketed	Oral
Methacycline	Rondomycin	1965	Marketed	Oral
Doxycycline	Vibramycin	1967	Marketed	Oral and parenteral
Minocycline	Minocin	1972	Marketed	Oral and parenteral
Tertiary-butylglycylamido-minocycline	Tigilcycline	1993	Phase II clinical trials	

From: Chopra & Roberts 2002

Resistance mechanisms

Efflux proteins: Genes for *tet* efflux genes code for membrane-associated proteins that remove tetracycline from the cell. Efflux genes occur in Gram-positive and Gram-negative species. Most of the efflux proteins confer resistance to tetracycline but not minocycline or glycylcyclines. In contrast, the Gram-negative *tet(B)* gene confers resistance to both tetracycline and minocycline but not glycylcyclines (Chopra *et al.* 1992; Testa *et al.* 1993).

Ribosomal protection proteins: These are cytoplasmic proteins that protect the ribosome against tetracycline and also confer resistance to doxycycline and minocycline. Compared to tetracycline efflux proteins they confer resistance to a wider spectrum of tetracyclines, with the exception of *tet(B)*.

Enzymatic inactivation of tetracycline: The only example described to date for inactivation of tetracycline due to enzymatic modification is the product of the *tet(X)* gene (Speer *et al.* 1991). The gene was discovered as part of two closely related *Bacteroides* spp. transposons and has not been found outside the genus *Bacteroides*.

Other mechanisms of resistance: The *tet(U)* gene of *Enterococcus faecium* confers low-level tetracycline resistance (Ridenhour *et al.* 1996). The gene sequence does not exhibit significant homologies to efflux protein genes or ribosomal protection proteins. The *otr(C)* gene of *Streptomyces* has not undergone sequence analysis as yet, and the nature of the protection mechanism is subject to speculation (Chopra & Roberts 2001).

To evaluate the feasibility of PCR-based detection of tetracycline resistance genes, all available *tet* resistance gene sequences were retrieved from the GenBank and EMBL databases through the Australian National Genomic Information Service (ANGIS). The efflux protein genes *tet(A)*, *tet(B)*, *tet(D)*, *tet(G)* and *tet(L)*, and the ribosomal protection protein genes *tet(M)* and *tet(O)* were identified as the most frequently occurring genes that have been found in a variety of Gram-positive and Gram-negative species.

Table 3. Mechanism of resistance for *tet* and *otr* genes^a

Genes
Efflux
<i>tet</i> (A), <i>tet</i> (B), <i>tet</i> (C), <i>tet</i> (D), <i>tet</i> (E), <i>tet</i> (G), <i>tet</i> (H), <i>tet</i> (I), <i>tet</i> (J)
<i>tet</i> (Z) <i>tet</i> (30) ^b , <i>tet</i> (30) ^b
<i>tet</i> (K), <i>tet</i> (L)
<i>otr</i> (B), <i>tcr</i> 3 ^c
<i>tetP</i> (A)
<i>tet</i> (V)
<i>tet</i> (Y)
Ribosomal protection
<i>tet</i> (M), <i>tet</i> (O), <i>tet</i> (S), <i>tet</i> (W)
<i>tet</i> (Q), <i>tet</i> (T)
<i>otr</i> (A), <i>tet</i> (PB) ^d , <i>tet</i> ^e
Enzymatic
<i>tet</i> (X)
Unknown ^e
<i>tet</i> (U), <i>otr</i> (C)

From: Chopra & Roberts 2001

^a Grouped according to McMurry and Levy, 2000

^b First numbered genes (Levy *et al.* 1999)

^c Genes have not been given new designations (Levy *et al.* 1999)

^d *tetP*(B) is not found alone, and *tetP*(A) and *tetP*(B) are counted as one gene (Lytras & Rood 1996; Sloan *et al.* 1994)

^e *tet*(U) has been sequenced but does not appear to be related to either efflux or ribosomal protection proteins; *otr*(C) has not been sequenced (Pang *et al.* 1994; Ridenhour *et al.* 1996)

Sequences of these genes were extracted from the databases and subjected to comparative sequence analysis via multiple sequence alignment. Amplification primers were derived from the alignments, and PCR assays for rapid detection of the genes were developed. The assays were tested on eight tetracycline resistant strains and three strains that are susceptible to tetracycline. Putative resistance genes were detected in seven of the eight resistant strains.

2. Materials and Methods

2.1 Materials

2.1.1 Amplification primers

Primers for PCR amplification were produced by GeneWorks, Thebarton, South Australia.

2.1.2 Bacterial strains and media

Ten strains were obtained from the Australian Collection of Microorganisms, Department of Microbiology, University of Queensland, Brisbane, and from Pathcentre Nedlands, Department of Microbiology and Infectious Diseases, Perth. Two additional strains were isolated from skin swabs from a healthy person. Tryptone soya agar, Mueller-Hinton agar and defibrinated sheep blood were purchased from Oxoid Australia Pty. Ltd. Tetracycline hydrochloride was from Sigma-Aldrich.

2.1.3 DNA isolation

Calcium chloride, ethylenediamine-tetraacetic acid (EDTA), glycerol, isoamylalcohol, lauryl sulfate (SDS), lysozyme, phenol, phenol-chloroform and sodium acetate were purchased from Sigma-Aldrich. Thermostable proteinase K (fungal) was from Invitrogen.

2.1.4 Real-time PCR

The LightCycler – FastStart DNA Master SYBR Green I was purchased from Roche Diagnostics.

2.1.5 Conventional PCR

Platinum Taq DNA polymerase and 1 kb ladder were from Invitrogen. PCR grade deoxy-nucleotide-triphosphates (dNTPs), molecular screening (MS) agarose and molecular size standard XIV were supplied by Roche Diagnostics. Ethidium bromide solution was purchased from Continental Lab Products.

2.1.6 Thermal cyclers

Real-time PCRs with the LightCycler – FastStart DNA Master SYBR Green I were run on the Ruggedized Advanced Pathogen Detection Device (R.A.P.I.D.TM, Idaho Technology Inc., Salt Lake City, Utah, US). A Thermal Cycler PTC 200 (MJ Research, Massachusetts, US) was used for conventional PCR.

2.2 Methods

2.2.1 Susceptibility testing of strains

Bacterial strains used in this study are listed in table 4. Cells were grown on Mueller-Hinton agar containing 10, 20, 25 and 30 µg/ml tetracycline (stock solution 5 mg/ml in ethanol). Plates were incubated at 37°C and examined after 20 h. Controls were grown on Mueller-Hinton agar without tetracycline and incubated as above.

Table 4. Bacterial strains

Strain No	Strain ID	Species	Source	tet Resistance ^a
1	1016486	<i>Burkholderia cepacia</i>	PathCentre Perth	+
2	ATCC25416	<i>Burkholderia cepacia</i>	ACM, University of Qld.	+
3	ACM2193	<i>Erwinia chrysanthemi</i>	ACM, University of Qld.	-
4	1015932P	<i>Staphylococcus aureus</i>	PathCentre Perth	+
5	1016722R	<i>Staphylococcus aureus</i>	PathCentre Perth	+
6	1016674U	<i>Streptococcus</i> Group B	PathCentre Perth	+
7	4553874B	<i>Streptococcus</i> Group B	PathCentre Perth	+
8	ATCC9144	<i>Staphylococcus aureus</i>	ACM, University of Qld.	-
9	ATCC9290	<i>Shigella sonnei</i>	ACM, University of Qld.	-
10	SA	<i>Streptococcus</i> sp. ^b	CBRN DC	+
11	SB	<i>Streptococcus</i> sp. ^b	CBRN DC	+

^a: maximum concentration tested was 30 µg/ml

^b: species identity based on morphology only

2.2.2 DNA isolation

Cells were grown overnight on tryptone soya agar supplemented with 7 % defibrinated sheep blood. Approximately 10 colonies were resuspended in 500 µl TE buffer (10 mM Tris·HCl, 1 mM EDTA, pH 8.0) and washed 3 times in 500 µl TE buffer. The final cell pellet was resuspended in 500 µl TE buffer containing 1 µg/µl lysozyme (stock solution 50 mg/ml in distilled water) and incubated at 37°C for 1 h. SDS and proteinase K (stock solution 20 mg/ml in 10 mM Tris·HCl, 20 mM CaCl₂, 50 % glycerol (v/v), pH 7.5) were added to a final concentration of 0.5 % (v/v) and 50 µg/ml, respectively. Cells were incubated at 65°C for 1 h and the lysate was extracted with equal volumes of phenol, phenol : chloroform (24 : 1) and chloroform : isoamylalcohol (25 : 24 : 1). Nucleic acids were precipitated with sodium acetate/ethanol and dissolved in 250 µl distilled water. Nucleic acid concentration was determined spectrophotometrically at 260 nm.

2.2.3 Design of PCR amplification primers

Sequence data were obtained and analysed using the facilities of the Australian National Genomic Information Service (ANGIS; see Littlejohn 2000). A total of 677 available tetracycline resistance gene sequences was retrieved from the databases of EMBL and GenBank. Duplicate entries were eliminated, and sequences containing more than the tetracycline resistance gene information were truncated. Sequences representing tetracycline resistance gene classes as described to date (Chopra & Roberts 2001) were subjected to comparative sequence analysis

through the FASTA program in order to identify related sequences. Sequences of each tetracycline resistance gene class that displayed significant sequence homologies (>75 %) and occurred in three or more different bacterial species were aligned using the program clustalW. Amplification primers for the efflux genes *tet*(A), *tet*(B), *tet*(D), *tet*(G) and *tet*(L), and for the ribosomal protection protein genes *tet*(M) and *tet*(O) were derived from the alignments. Primers for amplification of a portion of the 16S ribosomal DNA (Stackebrandt & Charfreitag 1990) were used in positive control PCRs to ensure that negative results were not due to insufficient DNA quality. Primer sequences, their target positions and melting points (T_m) are shown in table 5.

Table 5. PCR amplification primers

Primer	Sequence ^a	T_m (°C)	Position ^b
<i>tet</i> (A) forward	GTCGCGCTCGACGCTGTCGGCATCGGCC	72	969-996
<i>tet</i> (A) reverse	CCGGTGATGCCGGCCACGATCCGCCCG	72	1232-1258
<i>tet</i> (B) forward	GGCCAAATTCCCGCAACGGTGTGGTGC	67	1188-1215
<i>tet</i> (B) reverse	GCCAATAACACCGGTTGCATTGGTAAGGC	63	1537-1564
<i>tet</i> (D) forward	GCTGGCGCTGTATGCGGTGATGCAGGTC	67	105-132
<i>tet</i> (D) reverse	GCCGGCCCCGCAATCAGCCCCGCCACC	72	382-407
<i>tet</i> (G) forward	CGGCCAAGTGCCTGC[GA]GCCCTATGGGTC	69	1772-1979
<i>tet</i> (G) reverse	CGGGAACACCATCCATCCCTGCGTGGC	67	1986-2012
<i>tet</i> (L) forward	GGAAC[AC]ATGAGTGT[GT]ATT[AG]TTTT[CT]GG	58	2584-2609
<i>tet</i> (L) reverse	CCTACAATTGC[TA]ATACC[CT]GTTCCCTCTG	61	2866-2894
<i>tet</i> (M) forward	CC[TA]AC[AT]GTCATTATATGGA[GA]AGACC	56	1258-1283
<i>tet</i> (M) reverse	CGAAAATCTGCTGG[CGA]GTACT[GA]ACAGGGC	64	1535-1562
<i>tet</i> (O) forward	CCGCCAAATCTTCTGGGCTTCTGTCGG	66	1515-1544
<i>tet</i> (O) reverse	CGCCCCTGAGAGATATTCCCTGCGGTGC	66	1842-1868
16S forward	GAGTTTGATC[AC]GGCTCAG	50	11-29
16S reverse	G[AT]ATTACCGCGGC[GT]GCTG	54	519-536

^a: bases in [] indicate degenerate primer positions

^b: position numbers for tetracycline resistance gene primers are according to the core sequence of the alignment (see appendix); position numbers for 16S rDNA primers are according to Brosius *et al.* 1978

2.2.4 Real-time PCR

Reactions were run on a R.A.P.I.D.TM with the LightCycler - FastStart DNA Master SYBR Green I (Roche Diagnostics) in volumes of 20 μ l. Positive control reactions for amplification of a 526 base pair (bp) fragment of the 16S rDNA contained 0.05 μ M forward and reverse primer, 4 mM MgCl₂, 10 ng DNA and 2 μ l 10x reaction buffer. The temperature profile comprised initial denaturation at 95°C for 10 min followed by 40 cycles of primer annealing at 50°C for 10 sec, primer extension at 72°C for 20 sec and denaturation at 95°C for 10 sec at the maximum temperature transition rate of 20°C/sec, with acquisition of the fluorescence signal at the end of each primer extension. Completed reactions were subjected to melting curve analysis in order to enable discrimination of specific products and primer-dimers. The temperature profile for melting curve analysis was 95°C for 0 sec (instrument goes to next target temperature once set temperature is reached), 50°C for 45 sec and heating to 94°C at a temperature transition rate of 0.1°C/sec under constant acquisition of the fluorescence signal. Upon completion of the melting curve samples were cooled to 40°C, and melting curve data were analysed. Duplicates of each reaction were analysed on ethidium bromide stained

agarose gels with the 1 kb ladder (Invitrogen) and molecular size standard XIX (Roche Diagnostics).

Reactions for amplification of tetracycline resistance gene fragments contained 0.2 μ M forward and reverse primer, 4 mM MgCl₂, 10 ng DNA and 2 μ l 10x reaction buffer. After initial denaturation at 95°C for 10 min, the following temperature profiles were applied for different target genes over 40 amplification cycles:

tet(A) - Primer annealing at 67°C for 5 sec, primer extension at 72°C for 10 sec and denaturation at 95°C for 10 sec.

tet(B) - Primer annealing at 63°C for 5 sec, primer extension at 72°C for 15 sec and denaturation at 95°C for 10 sec.

tet(D) - Primer annealing at 65°C for 5 sec, primer extension at 72°C for 20 sec and denaturation at 95°C for 10 sec.

tet(G) - Primer annealing at 63°C for 5 sec, primer extension at 72 °C for 15 sec and denaturation at 95°C for 10 sec.

tet(L) - Primer annealing at 52°C for 5 sec, primer extension at 72°C for 15 sec and denaturation at 95°C for 10 sec.

tet(M) - Primer annealing at 52°C for 5 sec, primer extension at 72°C for 15 sec and denaturation at 95°C for 10 sec.

tet(O) - Primer annealing at 62°C for 5 sec, primer extension at 72°C for 15 sec and denaturation at 95°C for 10 sec.

Melting curve analysis and agarose gel electrophoresis was done as above.

2.2.5 Conventional PCR

Reactions were run on a Thermal Cycler PTC 200 (MJ Research) in thin walled PCR tubes with reaction volumes of 50 μ l. Both positive control reactions for 16S rDNA amplification and PCRs for amplification of tetracycline resistance gene fragments contained 0.5 μ M forward and reverse amplification primer, 3 mM MgCl₂, 200 μ M of each dNTP, 1 unit Platinum® Taq DNA polymerase, 100 ng DNA and 5 μ l 10x reaction buffer. The temperature profile for 16S rDNA amplification comprised initial denaturation at 94°C for 2 min followed by 5 cycles of primer annealing at 53°C for 30 sec, primer extension at 72°C for 40 sec and denaturation at 94°C for 20 sec, 25 cycles of annealing at 48°C for 20 sec, primer extension at 72°C for 40 sec and denaturation at 94°C for 20 sec and a final extension step with annealing at 48°C for 20 sec and primer extension at 72°C for 2 min. Reactions were cooled to 4°C prior to analysing 20 μ l aliquots on ethidium bromide stained 1.5% molecular screening (MS) agarose (Roche Diagnostics) gels. Size standards were the 1 kb ladder (Invitrogen) and molecular size standard XIV (Roche Diagnostics). The size of the amplification products as determined by comparison to the standards was estimated to be correct within \pm 25 bp.

After initial denaturation at 94°C for 2 min the following temperature profiles for amplification of tetracycline resistance gene fragments were applied over 40 cycles:

tet(A) - Primer annealing at 63°C for 30 sec, primer extension at 72°C for 30 sec and denaturation at 94°C for 30 sec.

tet(B) – Primer annealing at 58°C for 30 sec, primer extension at 72°C for 30 sec and denaturation at 94°C for 30 sec.

tet(D) – Primer annealing at 64°C for 30 sec, primer extension at 72°C for 40 sec and denaturation at 94°C for 30 sec.

tet(G) – Primer annealing at 63°C for 30 sec, primer extension at 72°C for 30 sec and denaturation at 94°C for 30 sec.

tet(L) – Primer annealing at 52°C for 30 sec, primer extension at 72°C for 30 sec and denaturation at 94°C for 30 sec.

tet(M) – Primer annealing at 52°C for 30 sec, primer extension at 72°C for 30 sec and denaturation at 94°C for 30 sec.

tet(O) – Primer annealing at 62°C for 30 sec, primer extension at 72°C for 30 sec and denaturation at 94°C for 30 sec.

After a final extension step with annealing for 30 sec at the corresponding temperature for each assay and primer extension at 72°C for 2 min, reactions were cooled to 4°C and analysed on agarose gels as above.

3. Results and Discussion

The positive control primers for amplification of a portion of the 16S rDNA generated a product of the desired size without formation of non-specific products as determined by agarose gel electrophoresis from all DNAs examined in this study. The theoretically expected size of 526 bp is based on the *E. coli* numbering system (Brosius *et al.* 1979). The actual amplicon size may vary by several base pairs due to insertions or deletions in the 16S rDNA of different strains. When used in real-time PCR, the amplification primers caused excessive primer-dimer formation in initial experiments (data not shown) but yielded satisfactory results when the primer concentration was limited to 0.05 µM and a relatively high annealing temperature of 53°C, 3°C above the T_m of the forward primer and 1°C below the T_m of the reverse primer, was applied. An amplification profile and a melting peak profile of a positive control PCR are shown in figure 1 and 2. Positive real-time PCRs were subjected to secondary analysis on ethidium bromide stained agarose gels to confirm the presence of amplification products of the correct length. An agarose gel for secondary analysis of control PCRs is shown in figure 3.

The results of conventional PCRs with the amplification primer pairs for detection of tetracycline resistance genes, expected amplicon lengths and lengths of the amplicons obtained are shown in table 6.

Table 6. Results of *tet* resistance gene PCRs

	<i>tet</i> Resistance Gene/Expected Product Length						
	tet(A) 300 bp	tet(B) 377 bp	tet(D) 303 bp	tet(G) 241 bp	tet(L) 311 bp	tet(M) 305 bp	tet(O) 354 bp
Strain							
<i>Burkholderia cepacia</i> 1016486*	300 bp	-	1300 bp 1200 bp 1100 bp	-	-	700 bp	-
<i>Burkholderia cepacia</i> ATCC25416*	550 bp	-	200 bp	-	-	-	-
<i>Erwinia chrysanthemi</i> ACM2193-	210 bp 190 bp	-	500 bp 190 bp	-	-	-	-
<i>Staphylococcus aureus</i> 1015932P*	-	-	-	-	-	305 bp	354 bp
<i>Staphylococcus aureus</i> 1016722R*	-	-	-	-	-	305 bp	354 bp
<i>Streptococcus</i> Group B 1016674U*	-	-	-	-	-	305 bp	354 bp
<i>Streptococcus</i> Group B 4553874B*	-	-	-	-	-	305 bp	354 bp
<i>Staphylococcus aureus</i> ATCC9144-	-	-	-	-	-	-	-
<i>Shigella sonnei</i> ATCC9290-	-	-	500 bp	-	-	1000 bp 950 bp	-
<i>Streptococcus</i> sp. Isolate 'SA'*	-	-	-	-	311 bp	305 bp	-
<i>Streptococcus</i> sp. Isolate 'SB'*	-	-	-	-	-	305 bp	-

*: Strain is resistant to 30 µg/ml tetracycline

-: Strain is susceptible to tetracycline $\geq 10 \mu\text{g}/\text{ml}$

Real-time PCR was of very limited use. The fluorescence amplification profiles indicated positive reactions with very few exceptions (see figure 4, 5, and 6) as significant primer-dimer formation occurred in most reactions. The main objective of the amplification primer design was high specificity with the lowest possible number of degenerate bases, and it was expected that the resulting relatively long primers (average of 28 bases) with high T_m values are not particularly well suited for real-time PCR. Further, in many cases the melting curve analysis failed to identify specific amplification products that were detected unambiguously by agarose gel electrophoresis, and some melting peaks that were interpreted as specific product

formation showed to be due to primer-dimer formation when the reactions were analysed on agarose gels eg., no distinct amplicon bands were visible. These misinterpretations of melting peak data were due to the fact that no DNAs harbouring the resistance genes investigated here were available as 'true' positive controls and to the high T_m of the primer-dimers resulting from long amplification primers with a high G+C content.

No amplification products were detected for the resistance genes *tet(B)* and *tet(G)* [table 6]. Amplification products of the expected length as derived from the alignments used for primer design were only obtained from strains that showed resistance against tetracycline concentrations of up to 30 $\mu\text{g}/\text{ml}$. Two resistance genes, *tet(L)* and *tet(M)*, were detected in *Streptococcus* sp. isolate 'SA'. The result is in accord with published data. Both genes are present in isolates of the gram-negative genera *Fusobacterium* and *Veillonella*, and of the gram-positive genera *Actinomyces*, *Bacillus*, *Listeria*, *Staphylococcus*, *Clostridium*, *Peptostreptococcus*, *Enterococcus* and *Streptococcus* (Chopra & Roberts 2001). The presence of both ribosomal protection protein genes *tet(M)* and *tet(O)* in *Staphylococcus aureus* 1015932P, *Staphylococcus aureus* 1016722R, *Streptococcus* Group B 1016674U and *Streptococcus* Group B 4553874B is also in accord with current literature (Chopra & Roberts 2001). However, the sequences of these genes show significant homologies over a wide range of species. The sequence of the *Campylobacter jejuni* *tet(O)* gene, used in this study as the core sequence for the *tet(O)* alignment, shows an average homology of 75 % to the *tet(M)* sequences of *Bacillus* sp. AF491293, *Gardnerella vaginalis* U58985, *Neisseria meningitidis* X75073, *Streptococcus faecalis* X56353, *Staphylococcus aureus* AY057894, *Staphylococcus aureus* M21136 and *Ureaplasma urealyticum* U08812. Subsequently, it appears more likely that the amplification primers for *tet(M)* and *tet(O)* detect the same gene in four of the species investigated here rather than indicating the presence of two separate ribosomal protection protein genes. The high homologies of *tet(M)* and *tet(O)* also suggest that the current nomenclature of *tet* resistance genes described to date may require reviewing. An agarose gel showing all putative amplicons from tetracycline resistance genes as indicated by their correct (predicted) size is shown in figure 7.

Amplification products of incorrect size were obtained from the tetracycline resistant strains *Burkholderia cepacia* 1016486 and *Burkholderia cepacia* ATCC25416, and from the susceptible strains *Erwinia chrysanthemi* ACM2193 and *Shigella sonnei* ATCC9290. The genes for microbial antibiotic resistance are frequently linked to transposable elements, and insertions or deletions may occur, resulting in dysfunctional genes. However, a more plausible explanation for incorrect size products obtained with the amplification primers for the efflux protein genes *tet(A)* and *tet(D)* are the relatively high homologies with other efflux protein genes (Chopra & Roberts 2001). Tetracycline efflux proteins show structural similarities with efflux proteins for multiple-drug resistance, quaternary ammonium resistance, and chloramphenicol and quinolone resistance, including methylenomycin A (MetA) from *Streptomyces coelicolor*, aminotriazole transport (Atr1) from *Saccharomyces*, and arabinose transport from *Escherichia coli* (Levy 1992; Sheridan & Chopra 1991). Homology has also been described for an efflux protein (EfpA) from *Mycobacterium tuberculosis* (Doran *et al.* 1997).

4. Conclusions

The seven assays under evaluation detected putative tetracycline resistance genes in seven out of eight resistant isolates. None of the tetracycline susceptible strains yielded amplification products of the expected size as predicted from the multiple sequence alignments for design of the amplification primers.

The occurrence of incorrect size products is likely to be due to the homology of tetracycline efflux pump protein genes with other efflux pump protein genes as discussed above. Detection assays derived from this study will utilise either hybridisation probes or TaqMan™ probes. These probes essentially enable amplicon confirmation and provide increased assay specificity that should suffice to eliminate false positive reactions caused by the presence of significantly homologous efflux pump genes.

Future research will include sequence analysis to confirm the identity of the putative tetracycline resistance genes and of the incorrect size amplification products obtained from the assays for *tet*(A), *tet*(D) and *tet*(M). Further, the assays require extensive validation prior to routine application. This will include testing on an extended collection of tetracycline resistant and susceptible isolates and on background organisms that are present in the majority of samples analysed for the presence of potential BW agents. As an operational requirement of the assays it has to be ensured that positive results are not due to the presence of resistance genes in background organisms. As demonstrated in this study, the considerable diversity of tetracycline resistance genes necessitates the design of a number of assays to provide coverage of the most frequently occurring genes. The assays cannot be combined as a 'one tube' multiplex assay with the existing assays for specific agent detection due to limitations of the detection platforms and the number of amplification primer pairs that can be used in one assay. They are intended for secondary analysis eg., for samples that tested positive for a potential BW agent.

The experimental work described here is a 'proof of principle' regarding the feasibility of assay development for the rapid detection of tetracycline resistance genes. Successful transformation of conventional PCR assays into real-time format will require some modifications. Amplification primers and fluorescently labelled detection probes will be designed utilising the 'Beacon Designer' software package from Premier Biosoft (Palo Alto, CA, US), with TaqMan probes as the preferred detection chemistry. The amplicon length of the conventional assays varied from 241 to 377 bp. These relatively long amplicons were generated to gain sufficient sequence information that allows assessment of the variations in *tet* resistance genes for the successful design of real-time assays. The amplicon length for real-time assays will be restricted to a maximum of 100 bp to reduce the time required for the amplification profile to approximately 40 min. Real-time assays will be run on the SmartCycler (Cepheid; Sunnyvale, CA, US). In contrast to the R.A.P.I.D. the SmartCycler allows the detection of up to four TaqMan probes conjugated to different fluorescent labels in the same reaction and can be applied for multiplex assays that detect three *tet* resistance genes and contain an internal control.

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6. Figures

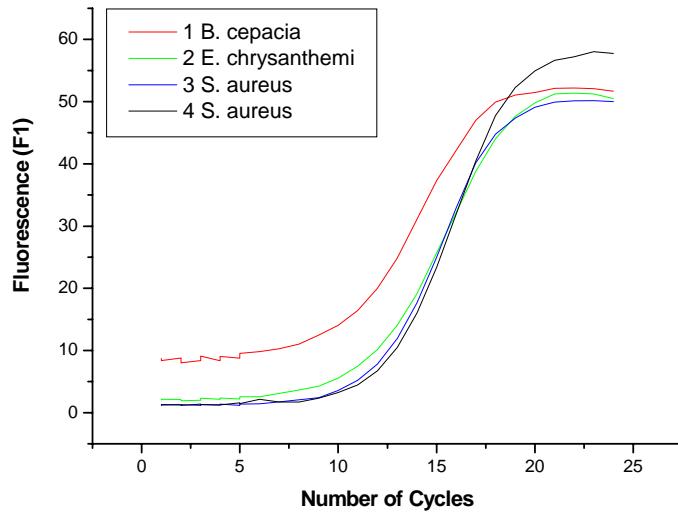


Figure 1. Fluorescence amplification profile of the 5'-16S rDNA region of strains 1 – 4

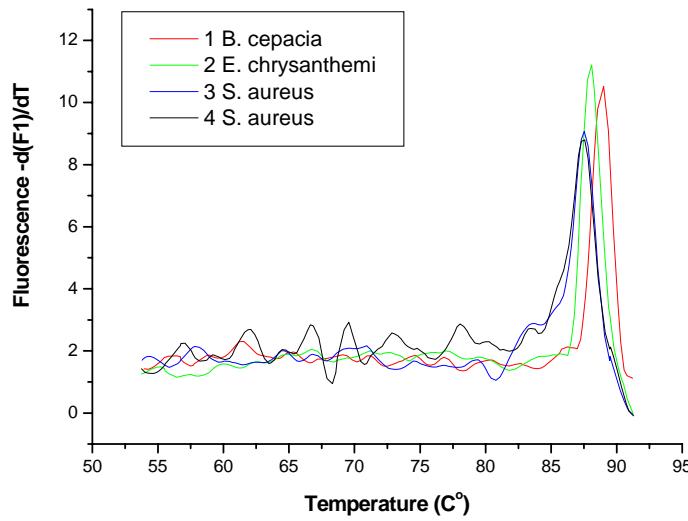


Figure 2. Melting peak profile of the 5'-16S rDNA region of strains 1 – 4. The melting peaks indicate homogeneous product formation and absence of primer-dimers. Differences in T_m are due to the variable regions within the 16S rDNA.

1 2 3 4 5

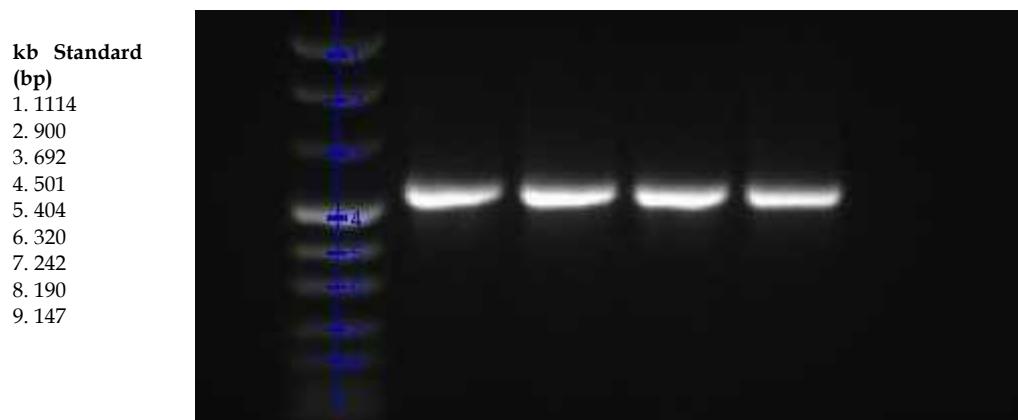


Figure 3. Agarose gel for secondary analysis of real-time PCRs shown in fig. 1 and 2. Lane 1-kb standard; lane 2-*Burkholderia cepacia*; lane 3-*Erwinia chrysanthemi*; lane 4-*Staphylococcus aureus*; Lane 5-*Staphylococcus aureus*

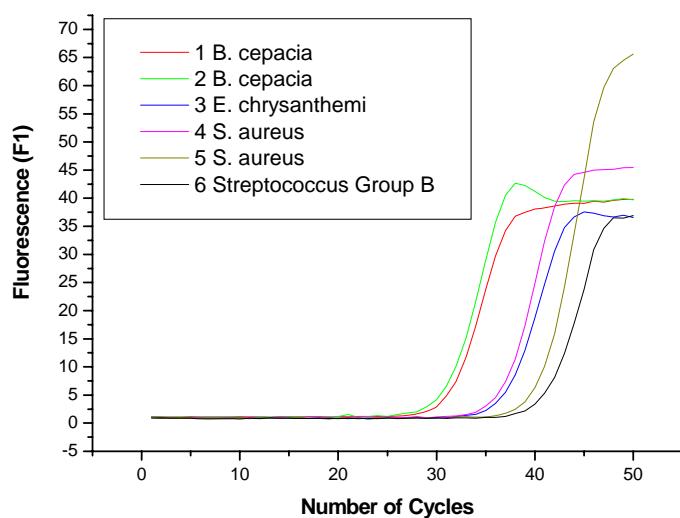


Figure 4. Fluorescence amplification profile of *tet(O)* for strains 1–6. The fluorescence curve of strains 1, 2 and 3 is due to primer-dimer formation only. No specific product formation occurred as determined by agarose gel electrophoresis.

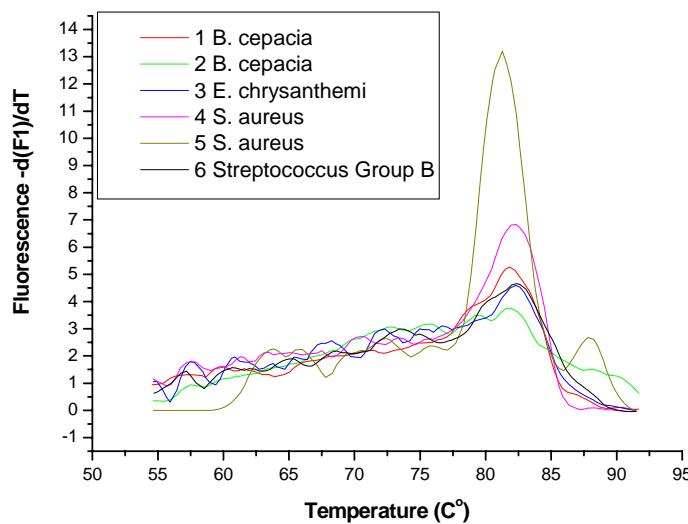


Figure 5. Melting peak profile of *tet(O)* amplification from strains 1 – 6. Agarose gel electrophoresis showed that specific amplicons were only generated from strains 4 – 6. In contrast, melting peaks indicate specific amplicons from strains 1, 4 and 5.

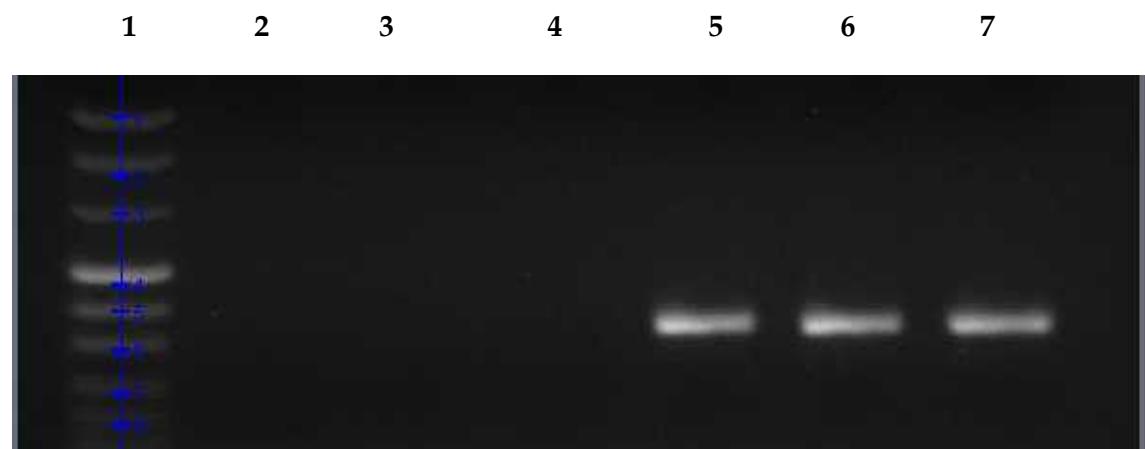


Figure 6. Agarose gel for secondary analysis of *tet(O)* amplification showing that specific amplicon was only generated in reactions 4, 5, and 6. Lane 1-kb standard (see fig. 3); lane 2-Burkholderia cepacia; lane 3-Burkholderia cepacia; lane 4-Erwinia chrysanthemi; lane 5-Staphylococcus aureus; lane 6-Staphylococcus aureus; lane 7-Streptococcus Group B

1 2 3 4 5 6 7 8 9 10 11 12 13

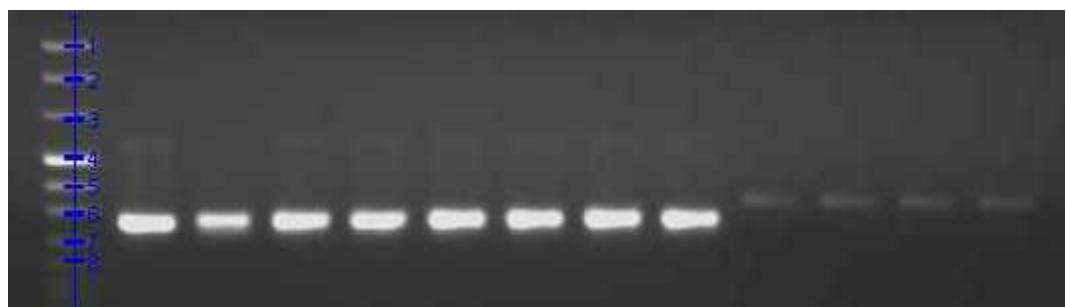


Figure 7. Agarose gel showing all amplicons displaying the expected length. Lane 1-kb standard (see fig. 3); lane 2-tetA, *Burkholderia cepacia*; lane 3-tetL, *Streptococcus* sp.; lane 4-tetM, *Staphylococcus aureus*; lane 5-tetM, *Staphylococcus aureus*; lane 6- tetM, *Streptococcus* Group B; lane 7-tetM, *Streptococcus* Group B; lane 8-tetM, *Streptococcus* sp.; lane 9-tetM, *Streptococcus* sp.; lane 10-tetO, *Staphylococcus aureus*; lane 11-tetO, *Staphylococcus aureus*; lane 12-tetO, *Streptococcus* sp.; lane 13-tetO, *Streptococcus* sp. The relative weak bands obtained from tet(O) amplification (lanes 10-13) indicate mismatches between target and amplification primers.

Appendix

Accession numbers and annotations for sequences used for comparative sequence analysis in multiple alignments to design *tet* resistance gene PCR primers

Efflux pump *tet*(A): **AJ307714***, *Escherichia coli* *tet*(A) gene; **AY196695**, *Acinetobacter baumanii* *tet*(A) genes; **X75761**, *Pseudomonas aeruginosa* *tet*(A) gene; **AJ419171**, *Escherichia coli* *tet*(A) gene; **AJ313332**, *Escherichia coli* *tet*(A) gene; **X61367**, gram-negative bacteria transposon Tn1721 *tet*(A) gene; **X00006**, *Escherichia coli* plasmid RP1 tetracycline resistance determinants; **AF534183**, *Shigella sonnei* plasmid pSS4 antimicrobial resistance determining region; **AF502943**, *Shigella sonnei* plasmid pSSTAV TetR class A variant; **AF497970**, *Shigella sonnei* plasmid pKKTET7; **AF542061**, *Salmonella enteritidis* expression vector pSY7K *tet*(A) gene; **Y19119**, *Salmonella enterica* serovar Dublin partial *tet*(A) gene

Efflux pump *tet*(B): **AB084245***, *Neisseria meningitidis* *tet*(B) gene; **AF162223**, *Shigella flexneri* transposon Tn10; **AF223162**, *Salmonella typhi* plasmid R27 transposon Tn10; **J01830**, transposon Tn10 tetracycline resistance and repressor genes *tet*(A) and *tet*(R); **AB084286**, *Neisseria meningitidis* *tet*(B) gene; **Y19113**, *Shigella flexneri* partial plasmid *tet*(B) gene; **AJ278685**, *Pasteurella aerogenes* plasmid pPAT2 *tet*(B) gene; **V00611**, transposon Tn10; **AJ277653**, *Escherichia coli* plasmid pGB1; **U81141**, *Shigella flexneri* multi-antibiotic resistance locus

Efflux pump *tet*(D): **Y19115***, *Aeromonas liquefaciens* partial plasmid *tet*(D) gene; **AF467077**, *Shigella flexneri* strain Gibbon32055 tetracycline resistance protein *tet*(A) gene; **AF467073**, *Shigella sonnei* strain 306 tetracycline resistance protein *tet*(A) gene; **AF467076**, *Shigella sonnei* strain PCRs-0612 tetracycline resistance protein *tet*(A) gene; **AF467075**, *Shigella sonnei* strain BL165 tetracycline resistance protein *tet*(A) gene; **AF467078**, *Shigella flexneri* strain SH4 tetracycline resistance protein *tet*(A) gene; **AF467072**, *Shigella sonnei* strain 252 tetracycline resistance protein *tet*(A) gene; **AF467074**, *Shigella sonnei* strain 119 tetracycline resistance protein *tet*(A) gene; **AF467072**, *Shigella sonnei* strain 305 tetracycline resistance protein *tet*(A) gene; **L06798**, plasmid pRA1 *tet*(D) gene; **X65876**, *Salmonella ordonez* gene class D *tet*(A)

Efflux pump *tet*(G): **AF133139***, *Pseudomonas* sp. Plasmid pPSTG1 tetracycline resistance protein *tet*(G) gene; **S52437**, *Vibrio anguillarum* plasmid pJA8122 Tet A (tetA (G) gene; **Y19118**, *Salmonella typhimurium* partial *tet*(G) gene; **AF119247**, *Salmonella typhimurium* DT104 tetracycline resistance protein *tet*(G) gene; **AF261825**, *Salmonella enterica* subsp. *enterica* serovar *typhimurium* genomic island I; **AF133140**, *Pseudomonas* sp. plasmid pPSTG2 tetracycline resistance protein *tet*(G) gene; **AF071555**, *Salmonella typhimurium* DT104 antibiotic resistance gene cluster; **Y19117**, *Salmonella typhimurium* partial *tet*(G) gene for tetracycline resistance protein; **AJ276217**, *Mannheimia haemolytica* partial *tet*(G) gene for tetracycline resistance protein class G;

Efflux pump *tet*(L): **M63891***, *Bacillus stearothermophilus* plasmid pTB19 tetracycline resistance protein *tet*(L) gene; **M34478**, *Bacillus thuringiensis* tetracycline resistance gene; **AY081910**, *Enterococcus faecium* tetracycline resistance protein *tet*(L) gene; **X15669**, *Streptococcus agalactiae* plasmid pMV158 *tet*(L) gene; **M63891**, *Bacillus stearothermophilus* plasmid pTB19 tetracycline

resistance protein *tet*(L) gene; **X60828**, *Staphylococcus hyicus* plasmid pSTE1 *tet* gene for tetracycline resistance protein; **M11036**, *Bacillus stearothermophilus* plasmid pTHT15 tetracycline resistance gene; **U38656**, *Staphylococcus aureus* tetracycline resistance plasmid pKH1 *tet* gene; **S67449**, *Staphylococcus aureus* plasmid pT181 tetracycline efflux protein *tet*(K); **M16217**, *Staphylococcus aureus* plasmid pNS1 encoding tetracycline resistance; **U38428**, *Staphylococcus aureus* tetracycline resistance plasmid pKH6; **U36910**, *Staphylococcus aureus* plasmid J3358; **J01764**, plasmid pT181; **S74032**, *Mycobacterium fortuitum* *tet*(K) tetracycline resistant mutant; **D12567**, *Bacillus subtilis* *tet*BSR gene; **X08034**, *Bacillus subtilis* GSY908 *tet* gene for tetracycline resistance; **U35229**, *Staphylococcus epidermidis* tetracycline resistance protein *tet*(L) gene

Ribosomal protection protein *tet*(M): AF491293*, *Bacillus* sp. R89 Tn916-like transposon ribosomal protection protein *tet*(M) gene; **X04388**, Streptococcal conjugative shuttle transposon Tn1545 *tet*(M) gene for tetracycline resistance; **AB064984**, *Clostridium septicum* gene for *tet*(M); **L12242**, *Neisseria gonorrhoeae* strain 2309 plasmid pOZ101; **AF440277**, *Lactobacillus plantarum* plasmid pMD5057; **X75073**, *Neisseria meningitidis* plasmid DNA for *tet*(M); **U58986**, *Gardnerella vaginalis* tetracycline resistance gene *tet*(M); **L12241**, *Neisseria gonorrhoeae* strain 6418 plasmid pOZ100 DNA sequence; **U58985**, *Gardnerella vaginalis* tetracycline resistance gene *tet*(M); **AF329848**, *Clostridium perfringens* transposon CW459*tet*(M); **M21136**, *Staphylococcus aureus* tetracycline resistance *tet*(M) gene; **AY057892**, *Staphylococcus aureus* strain 1802 tetracycline resistance protein *tet*(M) gene; **AY149596**, *Lactobacillus sakei* subsp. *sakei* isolate DG525 tetracycline resistance protein *tet*(M) gene; **U09422**, *Enterococcus faecalis* DS16 transposon Tn916 *tet*(M) gene; **X56353**, *Streptococcus faecalis* *tet*(M) 916 gene for tetracycline resistance; **M85225**, *Enterococcus faecalis* strain DS16 *tet*(M) gene; **X92947**, *Enterococcus faecalis* *tet*(M) gene; **U08812**, *Ureaplasma urealyticum* tetracycline resistance determinant *tet*(M) gene; **AB039845**, *Erysipelothrix rusiopathiae* gene for *tet*(M); **AY057894**, *Staphylococcus aureus* strain 2952 tetracycline resistance protein *tet*(M) gene; **L09756**, *Listeria monocytogenes* *tet* gene

Ribosomal protection protein *tet*(O): M18896*, *Campylobacter jejuni* *tet*(O) gene; **AY190525**, *Campylobacter jejuni* plasmid pCjA13 tetracycline resistance protein *tet*(O) gene; **M20925***, *Streptococcus mutans* tetracycline resistance *tet*(O-s) gene; **AJ295238**, *Clostridiaceae* strain K10 *tet*(32) gene for tetracycline resistance protein; **AY196921**, *Megasphaera elsdenii* strain 7-11 tetracycline resistance protein *tet* gene

*: core sequence of the alignment on which the numbering of the primer sequences (table 5) is based

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19. ABSTRACT The rapid spreading of bacterial antibiotic resistance and the lack of methods that would allow fast and accurate determination of the resistance profile of an infectious agent have become a major concern for public health. In the context of biological warfare or a bioterrorist attack, resistance against commonly used antibiotics would severely compromise the capabilities of the responsible agencies to protect large numbers of exposed individuals against a potentially lethal infection. In this study the possibility of developing polymerase chain reaction (PCR) assays for rapid detection of tetracycline resistance genes was evaluated. PCR assays were designed for seven resistance genes that frequently occur in Gram-positive and Gram-negative species and tested on eight tetracycline resistant clinical isolates. Positive results were obtained from seven of the eight species tested.				